Pulmonary Carcinogenicity of Inhaled Particles and the Maximum Tolerated Dose

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Chronic inhalation bioassays in rodents are used to assess pulmonary carcinogenicity for purposes of hazard identification and potentially for risk characterization. The influence of high experimental doses on tumor development has been recognized for some time and has led to the concept of maximum tolerated dose (MTD) for dose selection, with the highest dose being at the MTD. Exposure at the MTD should ensure that the animals are sufficiently challenged while at the same time the animal's normal longevity is not altered from effects other than carcinogenicity. A characteristic of exposure–dose–response relationships for chronically inhaled particles is that lung tumors are significantly increased only at high exposure levels, and that lung tumors are seen in rats only but not in mice or hamsters. This lung tumor response in rats is thought to be secondary to persistent alveolar inflammation, indicating that the MTD may have been exceeded. Thus, mechanisms of toxicity and carcinogenicity may be dose dependent and may not operate at lower doses that humans normally experience. Despite awareness of this problem, carcinogenicity bioassays that evaluate particulate compounds in rodents have not always been designed with the MTD concept in mind. This is due to several problems associated with determining an appropriate MTD for particle inhalation studies. One requirement for the MTD is that some toxicity should be observed. However, it is difficult to define what degree of toxic response is indicative of the MTD. For particle inhalation studies, various noncancer end points in addition to mortality and body weight gain have been considered as indicators of the MTD, i.e., pulmonary inflammation, increased epithelial cell proliferation, increased lung weight, impairment of particle clearance function, and significant histopathological findings at the end of a subchronic study. However, there is no general agreement about quantification of these end points to define the MTD. To determine whether pulmonary responses are indicative of the MTD, we suggest defining an MTD based on results of a multidose subchronic and chronic inhalation study with a known human particulate carcinogen, e.g., asbestos or crystalline silica. Quantification of effects in such a study using the noncancer end points listed above would identify a dose level without significant signs of toxicity at the end of the subchronic study. If this dose level still results in significant lung tumor incidence at the end of the chronic study. We will have a sound basis for characterizing the MTD and justifying its use in future particle inhalation studies. Also, a better understanding of cellular and molecular mechanisms of particle-induced lung tumors is needed to support the MTD concept. — *Environ Health Perspect* 105(Suppl 5):1347–1356 (1997)

Key words: nonfibrous particles, fibers, lung tumor, rodent, bioassay, inhalation, dose–response

Introduction

The rodent carcinogenicity assays generally involve dosing animals over a 2-year period. Historically, high dose levels have often been selected which could induce significant toxicity or even mortality from causes other than cancer. This problem has been recognized for some time, and guidelines were proposed to limit the highest dose in such assays to the maximum tolerated dose (MTD) (*1*). These guidelines were based mainly on results from the oral exposure route, and although the same principles apply to other routes of animal dosing, there are specific differences that must be considered when applying the concept of the MTD to chronic particle inhalation studies. In this brief review of the pulmonary carcinogenicity observed in particle inhalation studies with rodents and the underlying potential mechanistic events, we identify respective problems with the current MTD definition. We conclude with a suggestion for defining the MTD for particle inhalation studies.

Particle Inhalation Studies and High-dose Effects

Table 1 lists several nonfibrous and fibrous particles which were used in chronic inhalation studies in rats and which induced lung tumors in this species. This list includes particles of low cytotoxicity such as carbon black, talc, and TiO_2, as well as cytotoxic particles such as quartz and various types of asbestos. All of these particles are poorly or only moderately soluble in the lung. Although it is conceivable that mechanisms of lung tumor induction in rats differ between fibrous and nonfibrous particles, the studies with particles listed in Table 1 were performed with inhaled concentrations (up to 250 mg/m^3) much higher than those to which humans are exposed. One reason for using high exposures in these studies is to assure that the animals are adequately dosed so that during the short lifespan of a rat—compared to humans—the detection of potential carcinogenic effects is maximized. In the case of TiO_2, a difference between

Table 1. Chronic particle inhalation studies in rats that resulted in induction of lung tumors

Nonfibrous	Fibrous
Antimony trioxide	Amosite
Carbon black	Anthophyllite
Coal dust	Chrysotile
Diesel soot	Crocidolite
Nickel oxide	Palygorskite
Nickel subsulfide	Potassium titanate
Oil shale	Refractory ceramic fiber
Quartz	Silicon carbide
Talc	Tremolite
Titanium dioxide (pigment grade; ultrafine)	
Volcanic fly ash	

Data from Morrow et al. (*37*) and Oberdörster (*7*).

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Abbreviations used: AM, alveolar macrophage(s); MTD, maximum tolerated dose; PMN, polymorphonuclear cells; NTP, National Toxicology Program; SOD, superoxide dismutase; U.S. EPA, U.S. Environmental Protection Agency.

ultrafine TiO_2 particles (particle diameter ~20 nm) and pigment grade TiO_2 (particle diameter 200–300 nm) should be noted: An inhaled mass concentration more than an order of magnitude lower and respectively lower gravimetric lung burdens of the ultrafine particles compared to the larger sized TiO_2 resulted in similar lung tumor incidence (*2,3*). This result is of interest, as it suggests that the term dose should not be used narrowly as a gravimetric lung burden only, and that other particle parameters can be more appropriate dosemetrics as discussed below.

Based on the available data, our current knowledge of particle-induced lung tumors in rats can be summarized by stating that all inhaled particles—fibrous and nonfibrous—are likely to induce lung tumors in rats provided the particles are *a*) inhaled chronically at high concentrations, *b*) rat respirable, and *c*) of low *in vivo* solubility. The retained lung burden leading to induction of lung tumors can differ, depending on particle cytotoxicity, particle size, and particle shape, but is greater than 1 mg/g of lung for low toxicity particles. Although some of the particles listed in Table 1 have been characterized as confirmed human lung carcinogens, i.e., nickel sulfide, quartz, and various forms of asbestos (*4,5*), others were not found to be associated with an increase in lung tumors in exposed workers. Thus, the results of the rat studies raise the question as to whether these particles should be labeled as possible or even as probable human carcinogens. Underlying this problem is the fundamental question about the relevancy of the rat as an experimental animal for extrapolation of results of particle inhalation studies to humans, as the tumorigenic effect may be due to mechanisms that are only operative in the rat or operative only at high doses, which are not achieved in human lungs. The next section addresses these questions.

Exposure–Dose–Response Relationships in Rats

Multidose chronic inhalation studies in rats, using poorly soluble particles of low cytotoxicity, typically induced lung tumors at high concentrations only. Respective lung burdens were at a level characterized as particle overload of alveolar macrophages (AM), such that their physiological clearance function was severely impaired or had ceased completely (*6*). Chronic alveolar inflammation occurred as well. The presence of impaired AM-mediated particle clearance and chronic inflammation did not always result in a tumorigenic response in the rat studies, i.e., if these effects were only moderate. However, lung tumors were never found in rats when particle overload induced impaired clearance, and pulmonary inflammation were absent during chronic inhalation of particles (*7*). Respective dose–response relationships are consistent with a threshold dose above which lung tumors could be induced by a mechanism that may not be operative at lower doses.

Figure 1 depicts the result of the 2-year inhalation study in rats with pigment grade TiO_2 (particle size 200–300 nm) at exposure concentrations of 10, 50, and 250 mg/m^3 (*2*). For purposes of comparison with ultrafine TiO_2 (particle size ~20 nm) reported in another study (*3*), the exposure term in Figure 1 is expressed as exposure concentration × duration (g/m^3 × hr). For pigment grade TiO_2, lung tumor incidence was increased only at the highest concentration, with a lung burden of 665 mg per rat lung. The realization that this result in the rat is secondary to toxicity based on lung particle overload led the U.S. Environmental Protection Agency (U.S. EPA) to remove TiO_2 from its toxic release inventory (*8*). Since other rodent species do not show this response, the appropriateness of using the rat model in a cancer bioassay with inhaled particles was questioned. Doubts about human extrapolation from rat studies were reinforced by findings of high particulate lung burdens in coal miners [up to 40 mg/g lung (*9*)] that did not lead to an increased risk of lung cancer in this group of heavily exposed humans. (*5*). However, rats exposed to 200 mg/m^3 coal dust (Table 1) developed lung tumors, although the number of animals in the study was low (*10*).

Thus, it seems to be firmly established that rats respond with lung tumor induction at chronic high exposures to poorly soluble particles of low toxicity, which results in the retention of high lung doses. However, even low exposure concentrations to such particles can induce lung tumors in rats. As mentioned before and shown in Figure 1, a 2-year inhalation study by Heinrich et al. (*3*) showed that ultrafine TiO_2 can increase lung tumor incidence in rats at much lower exposures and respective lower gravimetric lung burdens than were observed with larger sized pigment grade TiO_2 in the study by Lee et al. (*2*). TiO_2 lung burdens were 665 mg (pigment grade TiO_2) and 39 mg (ultrafine TiO_2). The different particle sizes of TiO_2 in the two studies can explain their differing biological activities, as demonstrated in a subchronic inhalation study in rats comparing pulmonary effects of pigment grade TiO_2 and ultrafine TiO_2 (*11,12*). In this study, pulmonary inflammatory response and impairment of AM-mediated clearance correlated best with the surface area rather than the mass of the retained particles. Further, a dosimetric evaluation of particle-induced carcinogenic responses in the rat revealed that particle surface area rather than mass, volume, or number of the retained particles correlated best with lung tumor incidence (*13*). To use the appropriate dose parameter is therefore crucial, and particle surface area appears to be the best descriptor. The concept that the surface area is a more relevant dose parameter than the mass of poorly soluble particles is very plausible as it is the surface of an insoluble particle which interacts with cellular and subcellular structures to elicit biological responses.

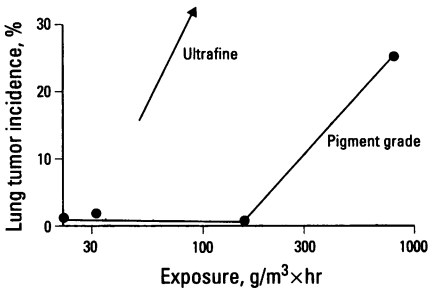

Figure 1. Exposure–response relationship of lung tumor incidence in rats after 2-year exposure to pigment grade TiO_2 particles (*2*), demonstrating a nonlinear response. Also shown is the result of a 2-year exposure to ultrafine TiO_2 particles (*3*).

Most recently, Driscoll (*14*) reinforced the surface area concept when he compared the rat lung tumor response in a number of particle inhalation studies with the mass or surface area of the retained particles. Only the surface area showed a highly significant correlation, as demonstrated in Figure 2. The ultrafine TiO_2 dose retained in rat lungs in the study by Heinrich et al. (*3*), expressed as particle surface area, is in the range of the high dose of pigment grade TiO_2 in the study by Lee et al. (*2*). Figure 2 demonstrates again that particle-induced lung tumors in rats are a phenomenon of high doses (dose expressed as particle surface area) and that a threshold may exist below which the retained dose of particle surface area is too low to induce tumors. Because the ratio of particle surface area to particle mass can differ greatly among

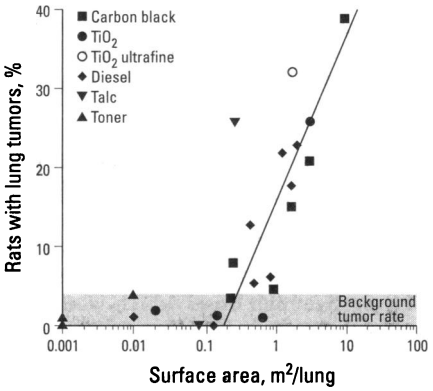

Figure 2. Dose–response relationship of lung tumor incidence in rats after chronic exposure to various particle types. Lung dose is expressed as surface area of retained particles (*14*). The shape of the curve is consistent with the existence of a threshold.

different types of poorly soluble particles of low toxicity, a high retained mass burden in the lung, although it may qualify as particle overload, is not necessarily associated with induction of lung tumors in rats. This is evidenced by the mid-dose of TiO_2 in the study of Lee et al. (*2*). The retained particle surface area is still low despite a high mass of 124 mg per rat lung.

We can infer from these arguments that particle mass is not the best dose parameter for particle-induced adverse pulmonary effects. Given this inference, the human data of high mass burdens in coal miners' lungs have to be viewed in a different light. If the dose concept of particle surface area is applicable to other species, including humans, then retained mass burdens of 40 mg/g human lung (as reported for coal miners [see above]) of a particulate compound with low surface area will translate into a low dose expressed as surface area. Thus, coal miners need not reach a critical dose in their lungs to be at an increased risk for lung cancer. Unfortunately, no data are available to convert reported mass burdens of inhaled and retained coal particles in coal miners' lungs to respective surface areas. However, data from Seixas et al. (*15*) and Burkhart et al. (*16*) suggest that the surface area of coal dust is not very high because of the rather coarse particle sizes of airborne coal mine dust, with means of the size distribution ranging from 5 to 10 μm. Thus, we have to be cautious in dismissing the rat as being an inappropriate model for human extrapolation unless we have more compelling evidence that specific mechanisms of tumor induction by particles operate in the rat only. It is clear, however, that poorly soluble particles of low cytotoxicity induce lung tumors in the rat only at high lung burdens.

In contrast to the rat, lung tumors have not been observed in mice and hamsters after chronic high-level exposures to nonfibrous particles of low solubility. Resistance to lung tumor induction in these two rodent species was evident in lung burdens of particles (i.e., talc, carbon black, diesel soot) that were as high as those in the rat studies, which resulted also in impaired clearance function of AM in mice and hamsters [reviewed by Oberdörster (*7*)]. However, the pulmonary inflammatory response elicited by particles was less pronounced in mice and hamsters, suggesting significant species differences in this response. With respect to inhaled fibrous particles, the hamster appears to be more likely to respond with mesothelioma than the rat but does not respond with lung tumors (*17*); the only chronic inhalation study in mice with fibers [chrysotile (*18*)] found that both lung tumors and mesotheliomas were induced.

In conclusion, lung tumor induction by inhalation of poorly soluble particles of low cytotoxicity (which showed negative results in genotoxicity assays) is highly species specific and only occurs at high dose levels. This may imply that a secondary mechanism of genotoxicity plays a role and that consequently certain defense mechanisms are better developed in one species compared to others and provide protection. Because the rat appears to be most sensitive with respect to lung tumor induction by high doses of inhaled particles, a discussion of mechanisms that are likely to be operative in this species at these high doses will be useful for an evaluation of the MTD and of the relevance of the rat model for humans.

Mechanism of Particle-induced Lung Tumors in Rats

Our present understanding of the cascade of events occurring in the rat lung during particle exposure is summarized in Figure 3. During chronic exposure to high concentrations of inhaled particles, phagocytosis by AM of particles deposited in the alveolar region results in AM activation, with release of a number of mediators including cytokines and chemokines. These mediators effect the recruitment of additional inflammatory cells, neutrophils (PMN), and macrophages, whose activation amplifies the existing inflammatory process through the release of additional inflammatory cytokines, growth factors, and reactive O_2- and N-species (*19,20*). The mitogenic activity of growth factors on epithelial target cells (e.g., type II cells) leads to an increase in their proliferation rate. In addition, an increase in target cell mutation rates via the action of reactive species (O_2-derived, N-derived, lipid peroxidation products) may occur, representing a secondary mechanism of particle-induced genotoxicity, with chronic alveolar inflammation playing a central role.

The inflammatory mechanism of particle-induced genotoxicity leading to lung tumors in rats is supported by a number of observations from both *in vitro* and *in vivo* studies. The knowledge that activated inflammatory cells can mediate a mutagenic response via released oxidants dates back to *in vitro* studies with human leukocytes and monocytes inducing bacterial mutations and modifications of DNA bases [reviewed by Weitzman and Gordon (*21*)]. First evidence supporting the inflammatory mechanism of particle-induced genotoxicity was provided by Driscoll (*14*) in *ex vivo* studies that showed that inflammatory cells obtained from the lungs of α-quartz-exposed rats are mutagenic to

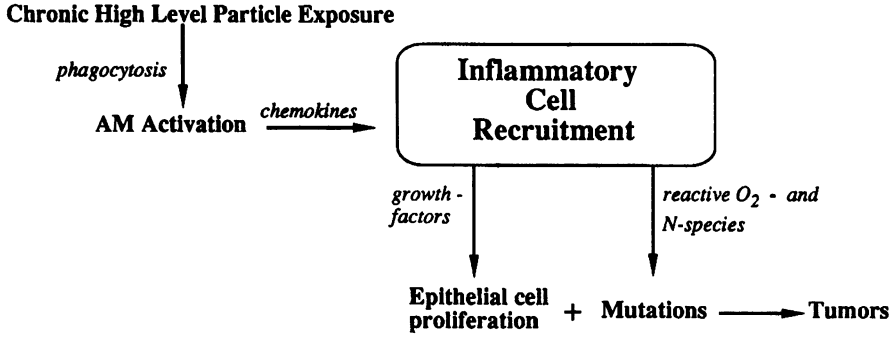

Figure 3. Chronic alveolar inflammation plays a central role in a suggested mechanistic sequence of lung tumor induction in rats by chronic inhalation of nongenotoxic particles.

rat alveolar epithelial cells and that the mutagenic effect could be attenuated by the addition of antioxidants. PMN appeared to be the most mutagenic inflammatory cells. Furthermore, a 3-month inhalation exposure of rats to carbon black particles confirmed that significantly increased *in vivo* mutation rates of alveolar epithelial cells occurred only in those groups that also showed a significant neutrophilic inflammation (*22*). Figure 4 shows similar results after intratracheal administration of high doses of different particle types to rats (*23*). The shape of the correlation between alveolar PMN response and alveolar epithelial cell mutation rates is consistent with the existence of a threshold, although it will be difficult to verify such threshold.

Conceivably, a threshold could be based on the balance between the reactive oxidants released by elicited inflammatory cells and specific antioxidant defenses associated with target cells. A number of adaptive responses will occur before the threshold dose is reached. During chronic particle exposures, these adaptive responses can include an increase in the number of AM to enhance phagocytosis capacity, as well as increased expression of antioxidants, antiinflammatory cytokines, metallothionein, and other protective mediators. Once these defense mechanisms are overwhelmed or exhausted adverse effects occur, potentially including mutagenicity carcinogenicity. The condition of lung particle overload in the rat is a prime example of clearance mechanisms being overwhelmed, resulting in subsequent effects secondary to high dose toxicity.

An explanation for the resistance of other rodent species to particle overload-associated

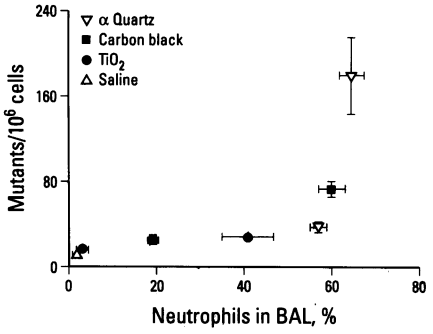

Figure 4. Correlation between alveolar inflammatory response, determined by the appearance of neutrophils in bronchoalveolar lavage and alveolar epithelial cell mutation rates in rats dosed with various particle types (*23*), supporting the hypothesis of inflammation-induced mutagenesis. BAL, bronchoalveolar lavage.

effects may lie partly in the levels of specific pulmonary antioxidant defenses, either baseline levels or inducible by a stimulus. Several authors have reported either higher pulmonary antioxidant levels in mice compared to rats (*24–26*), or in the case of extracellular SOD, a tetrameric form in mice and humans compared to a dimeric form in rats that leaves the rat pulmonary interstitium poorly protected enzymatically against superoxide radicals (*27*). Other potential mechanisms for the greater resistance of mice to particle-induced lung tumors may be related to differences in DNA repair mechanisms, apoptosis of transformed cells, or expression of antiinflammatory cytokines. With respect to the latter, IL-10 expression was reported to be significantly increased in lung lavage cells of mice after exposure to quartz (*28*), whereas IL-10 was not detectable after quartz treatment in rats (*29*). This suggests that the generally lower pulmonary inflammatory response in mice after particle exposure (*30–33*) may be due in part to greater expression of antiinflammatory cytokines.

Thus, the greater carcinogenic response to particles in rats compared with that of other rodent species may not be due to the inflammatory mechanism operating only in rats at high doses but may be due to differences in the level of the combined antioxidant and antiinflammatory defenses. At low particle doses in the lung, mechanisms of toxicity are well balanced by mechanisms of defense, but at high particle doses this balance becomes tilted toward toxic mechanisms, which in the rat eventually leads to tumor induction via oxidant-induced genotoxicity. The result is an apparent dose dependency of cellular mechanisms of toxicity and carcinogenicity. Principally, these toxic mechanisms could also operate in other species yet do not surface because of better developed defenses.

Molecular mechanisms of carcinogenicity have also been found to be dose dependent. Maronpot et al. (*34*) reviewed data showing that k-*ras* mutations in nitrosamine-induced lung and liver tumors occur at low but not at high doses. The apparent dose dependency of cellular and molecular mechanisms of carcinogenicity through the impact of toxicity challenges the basic assumption for carcinogenic risk assessment that tumors are induced by the same mechanism at low and high doses. The shape of the dose–response curve will be very flat in the low-dose region if mechanisms differ from low to high dose as discussed above. It is not known presently whether the suggested inflammatory mechanisms of secondary genotoxicity in particle-induced carcinogenesis described in Figure 3 for the rat is also operating in humans, but the goal of a chronic animal carcinogenicity assay is to identify agents that may be carcinogenic without the interference of significant toxicity. Thus, the need for dose limitation in a chronic (2 year) carcinogenicity bioassay is justified to avoid effects secondary to toxicity of excessive high doses which would not occur when anticipated exposures of humans are at much lower levels only. For selection of doses in such assays, applying the concept of MTD is principally very useful.

The term minimally toxic dose is also used interchangeably for the same acronym MTD, which may be viewed as unfortunate. However, conceptually, the underlying concept of dose limitation is not different between the two terms (*35*). Referring to MTD as minimally toxic dose may avoid the potential misunderstanding that "maximum" reflects the highest possibly achievable level (*36*). For particle inhalation studies, the key question is—When are levels of exposure and of resulting lung doses exceeding the MTD?

The MTD Concept

The MTD was initially defined as "... the highest dose of the test agent given during the chronic study that can be predicted not to alter the animals' normal longevity from effects other than carcinogenicity." (*1*). Additionally, it was stated that no clinical signs of toxicity or pathologic lesions or mortality unrelated to a neoplastic response should occur. The key factor in this early definition was survival of the animals to maximize study sensitivity. Another key consideration was body weight of the animals which was reflected in the statement that "...no more than a 10% body weight decrement, compared to the appropriate control group" should occur. Although body weight is a very sensitive indicator of toxicity in chronic cancer bioassays using oral dosing, which were mainly the basis for establishing these guidelines, chronic inhalation studies in rats even with highest concentrations of particles (e.g., 250 mg TiO_2/m^3) (*2*) did not result in changes in body weight (Figure 1). Thus, for chronic inhalation studies of particulate materials, the initial definition of MTD needs to be modified to take into account the difficulties in identifying adverse effects that are indicative of a minimally toxic dose. There is a need to validate for particle

inhalation studies the MTD concept that no significant toxicity unrelated to carcinogenicity should occur.

Use of the MTD as the highest dose (exposure level) in a chronic rodent bioassay assures that the animals are sufficiently challenged in order to identify agents that may be carcinogenic to humans, and also to identify potentially weak carcinogens. This means that hazard identification rather than risk characterization is the primary concern. Applying the concept of the MTD also provides the possibility to rank the carcinogenic potency of different compounds by comparing results from one study to another (*36*). The overall objective to maximize the likelihood of detecting a rodent carcinogen, and by implication a possible human carcinogen, when screening chemicals for their carcinogenic potential was the driving force for the MTD concept (*37*). The National Toxicology Program (NTP) prefers to refer to MTD as the minimally toxic dose (*35*) to avoid the misunderstanding that doses used are so excessive that animals barely survive. To decide whether the MTD has been achieved, major emphasis is placed on the histopathological evaluation of the target tissue. NTP (*38*) stated that at the MTD there should be no morphological evidence of toxicity that interferes with study interpretation. Increasingly, the process of dose selection is based on a mechanistic understanding of carcinogenesis. This is particularly important for agents like inhaled insoluble particles which, unlike soluble potentially reactive chemicals, are not metabolized in the lung but can be persistently retained, causing carcinogenicity secondary to toxicity as discussed previously. Thus, the selection of MTD for both fibrous and nonfibrous particles needs to include additional criteria to define the appropriate highest exposure concentration.

A recent U.S. Environmental Protection Agency (U.S. EPA)-sponsored workshop (*39*) titled "Chronic Inhalation Toxicity and Carcinogenicity Testing of Respirable Fibrous Particles" focused among other issues on the criteria for selecting the MTD for a chronic 2-year inhalation study to test carcinogenicity of fibers. In agreement with previous recommendations, it was suggested as a first step that the MTD for the chronic study should be based on the results of a subchronic multidose inhalation study with the fiber in question. This subchronic 3-month inhalation study, preferentially in rats, should show significant target organ responses at the highest exposure concentration to demonstrate that adequate dosing has been achieved. Rather than suggesting a highest exposure concentration in terms of number of fibers/cm^3, the workshop participants recommended that changes in the following parameters in response to fiber exposure be used for estimating the MTD:

- Lung weight
- Bronchoalveolar lavage parameters—cellular and biochemical
- Lung fiber burden normalized to the exposure concentration, with special consideration of fibers longer than 20 μm
- Quantification of target cell proliferation.
- Alveolar macrophage-mediated particle clearance
- Histopathology

Participants also recommended that these end points not be considered individually, but should be evaluated in the context of changes observed with the other parameters to evaluate thoroughly all effects occurring at the highest dose level. A further recommendation was to determine fiber biopersistence as this is an important characteristic for adverse long-term effects of fibers. The focus of the MTD discussion was on target organ responses. It should be noted that body weight as an indication for achieving the MTD was not included in the above list by the workshop participants, although this end point was an important one for the original MTD definition as mentioned before. Likewise, mortality was not included for evaluating the MTD, as a subchronic 3-month inhalation study with particles is unlikely to result in increased mortality, and if so, other signs of toxicity would be highly obvious.

Although this workshop was devoted to the evaluation of fibrous particles, the same end points for assessing that the MTD has been achieved can also be used for nonfibrous particles. The obvious difference will be a higher dose deposited in the lung if the nonfibrous particles are of a rather benign type such as TiO_2 or carbon black. However, the lung dose can also be low for cytotoxic nonfibrous particles such as crystalline silica. To obtain information on the persistence of specific end points, investigators should evaluate these end points again after a postexposure recovery period of 3 months. This will provide valuable information, especially if comparisons are made to fibrous or nonfibrous particles that serve as reference material (such as asbestos or crystalline silica), where changes will be very persistent.

Several difficulties arise when applying these recommendations for conducting a study. One problem is that the MTD to be used in the 2-year chronic study is determined from a 3-month subchronic study. Minimal changes in toxicity occurring at the 3-month time-point may well develop further so that by the end of a chronic study the MTD has been exceeded by far. Even the second dose level which may not lead to significant responses by 3 months may do so at the end of a 2-year study. Moreover, no recommendations were made as to the degree of a response for the above-listed specific end points. Thus, a major open question still is what is an acceptable response which would unequivocally be accepted as reflecting the MTD? Or, in other words, how much of a target organ response is enough?

Only a few attempts have been made to address this question. Guidelines for carcinogenicity testing (*40*) recognize the problem of defining the MTD for chronic cancer bioassays, but are not very specific with respect to quantifying responses. Bucher et al. (*35*) characterized acceptable and unacceptable histopathological lesions for different target organs (liver, kidney, lung) for identifying an MTD. In general, acceptable responses in the target organ signifying that the MTD has been achieved include minimal/mild changes, whereas exceeding the MTD and unacceptable changes include necrosis and more severe pathological lesions. Specifically for the lung, suggested acceptable changes should be minimal in terms of histiocytosis, focal inflammation, fibrosis, and hyperplasia. Although no unacceptable responses specific to the lung were given by Bucher et al. (*35*), it can be assumed that greater than minimal changes would fall into this category. However, as pointed out by McConnell (*36*), histopathology is a subjective science and, therefore, defining the degree of a change is a major problem: "One pathologist may interpret a given change as being moderate in severity, while another would interpret the lesion as mild in nature." Thus, it could be extremely difficult to agree not only on the magnitude of a pathological change but also on what degree of change is necessary to characterize a specific exposure as having reached the MTD. Ideally, an objective method is needed to quantify and calibrate specific end points which could be generally agreed on as reflecting the MTD.

Another difficulty previously mentioned is whether a subchronic study (13 weeks) is sufficient to estimate the MTD for a chronic study. Figure 5 shows the results of a 2-year inhalation study in rats exposed to diesel exhaust at three different concentrations (*41*). Bronchoalveolar lavages and evaluation of cellular and biochemical parameters were performed at 6, 12, and 24 months of exposure. As Figure 5 shows, there is a significant shift in the shape of the exposure–response (inflammatory response determined by neutrophils in lung lavage) relationship over time. The mid-concentration of 3.5 mg/m^3 showed only a slight increase in lavaged neutrophils of approximately 10% at 6 months of exposure, which is probably not indicative of an MTD being reached. However, at 12 months of exposure, lavaged neutrophils from rats of this exposure group had increased to 40%, which would likely be indicative of the MTD being reached or even exceeded; the neutrophil count in this group was even higher at 24 months of exposure. Only animals exposed to the mid and high concentrations in this particular study had increased lung tumor incidence (*42*), a result associated with a state of lung particle overload. Only the lowest concentration failed to induce lung tumors in the exposed rats, but even this concentration at the 24-month time point showed significant elevation of lavaged neutrophils (~27%). A subchronic 3-month study likely would not have given an indication that an MTD had been achieved based on the results of lavage analysis. Other end points mentioned above obviously would also need to be examined; however, the difficulty of using a 3-month study for predicting the MTD for the chronic study is quite obvious from this example.

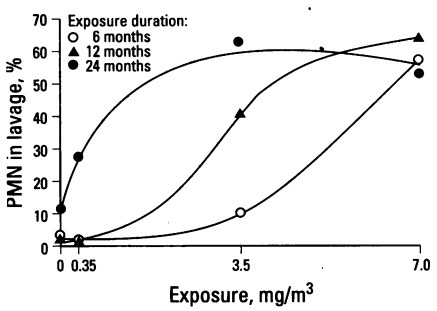

Figure 5. Exposure–response relationship in rats chronically exposed to three concentrations of diesel exhaust, with measurement of lung lavage neutrophils after 6, 12, and 24 months of exposure (*41*). PMN, lung lavage neutrophils. Note the shift in the shape of the response curves with increasing duration of exposure (*7*).

At present, no appropriate user friendly definition of MTD for particle inhalation studies is available. One problem relates to the fact that we do not know whether the mechanisms that lead to lung tumors in rats after inhalation of particles are also operating in humans. However, the knowledge that some particulate compounds are known to be human carcinogens and are also known to have induced lung tumors in long-term rat inhalation studies could be used to characterize the MTD, and to validate and even quantify the specific end points listed previously so that they can be used for future studies with unknown particulate compounds.

Defining the MTD for Particle Inhalation Studies

Numerous chronic rat inhalation studies with fibrous and nonfibrous particles have been and are still performed with highest exposure concentrations assumed to be at the MTD. This assumption is based on an evaluation of changes in the target organ including several of the noncarcinogenic parameters suggested in the previous section, i.e., histopathology, particle dosimetry, lung weight, bronchoalveolar lavage parameters, and also the function of AM-mediated particle clearance. Significant changes in these parameters are taken as an indication of the MTD. Although this is consistent with suggestions made by scientists from regulatory agencies, academie, and industry, it is still disputed as to whether in a given case the MTD has been achieved inspite of significant changes in the target organ. One problem is a lack of exposure–dose–response information for changes in these noncarcinogenic end points that can occur in rats after subchronic/chronic exposure to a known human particulate carcinogen.

To establish an appropriate definition of the MTD for particle inhalation studies and to calibrate specific non-cancer end points to be used for this purpose, we suggest performing a chronic multidose rat inhalation study with a known human particulate carcinogen. With respect to fibrous particles, asbestos would be a good choice, possibly using a long form of amosite as has been used in a recent chronic hamster inhalation study (*43*). With respect to a non-fibrous particle, crystalline silica would be an appropriate choice as it has just recently been labeled a human carcinogen based on sufficient evidence of carcinogenicity from epidemiology of occupationally exposed workers (*5*). Both amosite and crystalline silica have been shown in long-term rat inhalation studies to induce tumors in this species (*44–47*). However, none of these previous long-term inhalation studies was concerned with the concept of MTD, no prechronic studies were performed, nor were specific noncancer end points other than histopathology evaluated at the end of the 2-year time point. A characterization of specific pulmonary responses at different time points during a chronic study would therefore be needed to determine the degree of change in noncancer end points, which would indicate that an MTD was reached. If in the chronic study the known human particulate carcinogens induce lung tumors in the rat not only above but also below the MTD, this would confirm the validity of the underlying concept.

The suggested chronic study with long amosite or with crystalline silica could be designed as follows: Three or four concentrations of the particulate compounds should be used to expose male and female rats. Concentrations should range from approximately 10 to 200 fibers/cm^3 for the amosite, and it should be assured that enough of the long fibers greater than 20 µm are present (~20%). For the crystalline silica, concentrations may range from 0.1 to 3 mg/m^3, and the samples should consist of rat respirable particles. At 13 weeks of exposure, sufficient numbers of animals should be removed from the study so responses can be quantified regarding changes in lung weight, bronchoalveolar lavage parameters, lung burden of fibrous, and nonfibrous particles, alveolar–epithelial cell proliferation, AM-mediated particle clearance, and histopathology. Additional evaluations of the same end points may be performed at 6, 12, and 24 months of exposure. At the end of the 2-year study, the exposure–dose–response with respect to lung tumors and respective correlations to the noncancer end points measured during the study can be evaluated.

This is only a brief outline of the study; details still need to be worked out. Table 2 summarizes potential results in such study. It is assumed that at the end of the 2-year study a dose-dependent excess lung tumor incidence is observed so that the highest dose level satisfies the requirement of the MTD being reached or slightly exceeded. Toxicologically significant changes in noncancer end points at 3 months of exposure are assumed to be dose dependent, and three possible scenarios are listed in Table 2. Scenario 1 shows significant changes in noncancer end points only at the highest dose.

Table 2. Testing for pulmonary carcinogenicity of inhaled fibrous and non-fibrous particles in rats: principles of establishing an MTD benchmark by quantifying non-cancer end points (see text) in a multidose inhalation study with the known human particulate lung carcinogens amosite or crystalline SiO_2. Scenario 1 is the ideal outcome and would validate the present MTD concept.

		Inhaled particulate concentration		
		Low	Mid	High
Excess tumors at end of 2-year study		–	+	++ (> MTD)
Toxicologically significant changes in non-cancer end points at 3 months of exposure	Scenario 1	–	–	+
	Scenario 2	–	+	++
	Scenario 3	+	++	+++

In scenario 2 such changes are assumed to occur only in those groups in which tumors are seen, whereas in scenario 3 respective changes are assumed to be induced even at the lowest level where no tumors are seen. Only in scenario 1 did tumors occur below the MTD. Respective changes for the non-cancer end points can thus be quantitated (e.g., x-fold increase of neutrophils in lung lavage, or in target cell proliferation, etc.). The quantification of these changes at the 3-month time point could then be used as a benchmark for future studies with other non-fibrous and fibrous particles in a subchronic study designed to determine the MTD for a subsequent chronic study. This outcome of scenario 1 would validate the MTD concept with known human particulate carcinogens for the chronic rat bioassay.

If, however, amosite or crystalline silica induce only lung tumors in those groups that show significant changes in the non-cancer end points at 3 months and at later time points (scenario 2) or non-cancer changes occur even without excess tumors (Scenario 3), then we may have to reconsider the concept of MTD for particle inhalation studies. The implication of such findings would be either that mechanisms of amosite/silica-induced lung tumors between rats and humans are very different, or that humans in the positive epidemiologic studies were exposed at or above the human MTD. In the first case, the use of the rat as a model in cancer bioassays for inhaled particles needs to be reevaluated—the rat may have to be characterized as inappropriate for this purpose. In the second case, the MTD principle may need to be extended to include dose levels above the MTD in a rat–cancer bioassay with particles (scenarios 2 and 3 in Table 2). For example, the degree of an acceptable inflammatory or other toxic response may have to be greater than minimal as suggested by the present MTD concept.

Concluding Remarks

Assuming that the MTD concept has been validated by the studies outlined in the previous section and using the calibrated noncancer end points previously suggested, the carcinogenic potential of new particulate materials can be evaluated in a chronic cancer inhalation bioassay in the rat. However, regulators need to determine how to classify a particulate compound if a tumor response occurs only at and above MTD levels. Should such a compound be characterized as a possible human carcinogen? The U.S. EPA, when removing TiO_2 from its toxic substances inventory (*8*), used the weight of evidence approach to determine that lung tumors induced in rats at high levels of TiO_2 were secondary to toxicity, based on lung particle overload. Thus, it becomes important in such cases to evaluate mechanisms of lung toxicity and carcinogenicity for fibrous and nonfibrous particles, specifically comparing low versus high doses.

For an evaluation of secondary mechanisms such as inflammation-induced carcinogenesis (Figure 3), quantification of the specific end points of toxicity would also be very helpful. For example, the magnitude of an increase in inflammatory neutrophils in bronchoalveolar lavage and its significance for inducing mutational events in target cells may depend highly on the level of anti-inflammatory and antioxidant defenses. It may be possible to define an acceptable increase in lung lavage neutrophils (e.g., 20 or 30% of the lavaged cell population), which, although significant, may not be viewed as being above the MTD; i.e., levels of significance for toxic responses could be redefined.

It should be considered that for an evaluation of these noncancer end points only a small number of animals (~5) is necessary, whereas a carcinogenic effects study requires much greater numbers (50–100 per group) in order to detect significant tumorigenicity even at high doses. The definition of a high dose is by convention based on the gravimetric dose but should be redefined and based on the observed changes in non-cancer end points. For example, in the case of particles that have a strong inflammatory and fibrogenic potency, e.g., crystalline SiO_2, the markers of inflammation will be present at much lower mass doses than with TiO_2. However, at equal degrees of a chronic inflammatory response (above MTD), the lung tumor response in rats may well be the same for low dose SiO_2 and high dose TiO_2. What we do not know and what needs to be determined is whether the confirmed human carcinogen crystalline SiO_2 also induces lung tumors in rats at or below dose levels defined as the MTD in a validation study that is designed as suggested in this paper. If tumors are not induced at lower levels, the concept of the MTD for particle inhalation studies has to be reconsidered.

Being able to define the MTD based on the outcome of a validated study is obviously important for the long-term cancer bioassay. However, such long-term inhalation studies with particles are very time- and cost-consuming, and one future goal would be to develop short-term assays that will allow us to determine the carcinogenic potential of an unknown particulate compound. With respect to fibers, one approach presently being discussed involves combining in a short-term assay the evaluation of the biodurability of an unknown fiber with evaluation of specific end points of toxicity after an inhalation exposure as short as 5 days with subsequent sufficient time for observation. This short-term assay for fibers is based on the rationale that a fiber that is dissolved within a short period of time in the lung will no longer be fibrogenic or carcinogenic. The concept of biodurability for long-term chronic effects is also principally applicable to nonfibrous particles, e.g., crystalline silica (poorly soluble) versus amorphous silica (soluble in the lung). However, for the development of short-term assays, more research is still needed to gain insight into the mechanisms of particle-induced lung tumors so that future assays, either *in vivo* or *in vitro*, can be based on a better knowledge of cellular/molecular events.

Another important research area to be addressed in future studies is the significant species difference in response between rats on the one hand and mice and hamsters on the other . Why are mice and hamsters less susceptible to pulmonary inflammation, fibrosis, and carcinogenicity? With respect to the inflammatory mechanisms of lung tumor induction, a number of questions could be answered by evaluating species differences in the recruitment of neutrophils to the lung and their state of activation; investigating the

mutagenic potential of activated neutrophils from mice and hamsters to epithelial target cells; or determining the response of lung epithelial cells from mice and hamsters to mutagenic stimuli in comparison to rat epithelial cells. To answer these and other related questions would give important insight into reasons for the apparent resistance of some rodent species to lung tumor induction by particles. Finally, a significant gap in our knowledge needs to be closed by comparing rodent and human responses using lung cells from these species for evaluation of cellular and molecular events. The hope is that mechanistic knowledge gained from these studies will in the future permit us to replace the long-term cancer bioassay with acceptable short-term assays.

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